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Autoreactive B Cell Regulation: Peripheral Induction of Developmental Arrest by Lupus-Associated Autoantigens

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Summary

Anti-Sm and anti-ssDNA transgenic (Tg) mice were generated using the V_H-D-J_H rearrangement of an anti-Sm hybridoma of MRL/Mp-*lpr/lpr* origin. B cells of each specificity account for 15%–35% of the splenic repertoire, but no circulating anti-Sm or anti-ssDNA antibodies are detected. Most autoreactive cells exhibit an immature B cell phenotype and have short half-lives equivalent to those of non-Tg immature B cells. However, at least some anti-Sm B cells are functional, because immunization with murine snRNPs induces anti-Sm secretion. We propose that anti-Sm and anti-ssDNA are eliminated during the transition to mature B cells and that this late stage of tolerance induction is consequential to their spontaneous activation in murine lupus.

Introduction

Patients with systemic lupus erythematosus (SLE), an autoimmune syndrome that predominantly afflicts women, endure damage to various tissues including kidney and heart (Alarcon-Segovia, 1988). SLE is characterized by the production of anti-nuclear antibodies, some of which may directly cause tissue damage (Alarcon-Segovia, 1988; Shlomchik et al., 1994; Koren et al., 1995). In particular, anti-Sm and anti-ssDNA antibodies are diagnostic of SLE; however, the prevalence of even these specificities is less than 100%, because anti-ssDNA and anti-Sm are present in 70% and 25% of SLE patients, respectively (Tan, 1989). The factors that govern the prevalence of these responses are unknown. Our current understanding of responses to ssDNA and Sm stems from analysis of mice of various inbred strains that spontaneously develop an SLE-like disease (Cohen and Eisenberg, 1982; Boyer et al., 1985; Shores et al., 1986; Eisenberg et al., 1987a, 1987b, 1989, 1990; Shlomchik et al., 1987, 1990; Marion et al., 1990; Tillman et al., 1992, Reap et al., 1993; Bloom et al., 1994; Fatenejad et al., 1994; Radic and Weigert, 1994; Shan et al., 1994). MRL/Mp-*lpr/lpr* (MRL/lpr) mice are the only mice known to undergo a spontaneous anti-Sm response. In these mice, the prevalence is 25% and is independent of hereditary, maternal, and environmental factors (Eisenberg et al., 1987b).

We have examined V gene use and clonality of the anti-Sm response in MRL/lpr mice to understand the regulation of this response. Remarkably, we find that the anti-Sm and anti-DNA responses overlap extensively; many anti-Sm hybridomas bind ssDNA and even dsDNA (Bloom et al., 1993a, 1993b). Anti-Sm antibodies are encoded by multiple V_H and V_κ gene segment combinations and have no apparent restriction in V_HCDR3 (Bloom et al., 1993b), although in individual mice this response is oligoclonal and involves somatic mutation (Bloom et al., 1993a, 1993b; Retter et al., 1995). Although not required for specificity, somatic mutation can increase antibody affinity for Sm or DNA (Bloom et al., 1993a; Retter et al., 1996). These data rule out a requirement for a rare V(D)J gene combination or somatic mutation in controlling the prevalence of this response in MRL/lpr mice and indicate that both Sm and DNA can drive the anti-Sm response. The importance of this overlap to the development of autoimmunity has yet to be elucidated.

Anti-ssDNA-reactive B cells are generated in normal mice and regulated peripherally. Anti-ssDNA antibodies and B cells are detected in normal mice (Pisetsky and Caster, 1982; Conger et al., 1987), and immunization with foreign DNA can induce an anti-ssDNA response (Diamond and Scharff, 1984; Gilkeson et al., 1989a, 1989b, 1990, 1991; Pisetsky et al., 1990; Messina et al., 1991; Pisetsky, 1996; Ray et al., 1996). Moreover, anti-DNA Tg mice directly demonstrate peripheral regulation (Erikson et al, 1991; Offen et al., 1992; Tsao et al., 1993; Roark et al., 1995). We speculate that anti-Sm B cells are similarly regulated and are present in the periphery because immunization with Sm or peptides of Sm can, under certain circumstances, induce an anti-Sm response in normal mice (Shores et al., 1988; Fatenejad et al., 1993; Mamula et al., 1994; Bockenstedt et al., 1995).

To elucidate the regulatory mechanisms governing anti-Sm B cells in normal mice, we have generated μ chain transgenic mice using an unmutated, rearranged V_HJ558 gene from the MRL/lpr anti-Sm hybridoma 2-12 (Bloom et al., 1993b; Retter et al., 1996). This V_H is used by clonally independent anti-Sm hybridomas from multiple MRL/lpr mice and may be a common V_H in this response (Bloom et al., 1993b). Antibodies composed of this H chain and other L chains can bind with varying affinity to Sm, ssDNA, or both antigens (Retter et al., 1995). In addition, some combinations bind neither antigen. Thus, 2-12H Tg mice are expected to generate B cells specific for Sm, ssDNA, or both among a population of B cells that bind neither antigen and are presumably nonautoreactive.

We find that 2-12H chain Tg mice generate substantial numbers of B cells specific for Sm and ssDNA. About half of the splenic B cells, however, bind neither autoantigen, allowing us to examine the regulation of specific autoreactive cells in the context of nonautoreactive cells

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Table 1. Serum IgM Levels From Tg and Non-Tg Mice

	Tg	Non-Tg
IgM (mg/ml)	1.4 ± 0.789	3.9 ± 2.87
IgM^a (mg/ml)	0.377 ± 0.196	ND
IgM^b (mg/ml)	0.215 ± 0.131	3.4 ± 1.94

Data are from 10 Tg and 6 non-Tg mice.
ND, not detectable.

in the same mouse. Despite the presence of large numbers of autoreactive cells in the periphery, anti-Sm and anti-ssDNA serum autoantibody is not detectable, although in vitro lipopolysaccharide (LPS) stimulation of these cells induces secretion of anti-Sm and anti-ssDNA antibodies. Most anti-Sm- and anti-ssDNA-specific B cells appear to be immature B cells, and many appear to fail to progress beyond the immature phenotype, because the repertoires of the immature and mature subsets differ substantially in the frequency of autoreactive B cells. Surprisingly, immunization with mouse snRNPs induces an anti-Sm response, indicating that at least some anti-Sm B cells are functional. We propose a model of peripheral regulation in which many B cells of both specificities fail to become long-lived mature B cells.

Results

The 2-12H Transgene Supports B Cell Development

The 2-12H transgene construct used to generate the Tg mice (Retter et al., 1995) consists of the 2-12H V(D)J cloned upstream of the Igh^a allotype Cμ exons. The transgene encoded H chain excludes endogenous gene rearrangement and supports B cell development. Immunofluorescence analysis of spleen cells with anti-IgM^a and anti-IgM^b reveals that approximately 92% of the B cells in Tg mice express the transgene H chain and that the remainder express an endogenous H chain (data not shown). Approximately 3% stain for both allotypes, as compared to 0.35% of control B cells (data not shown). The level of serum IgM^a is approximately 10-fold lower than IgM of non-Tg mice but almost 2-fold higher than that of endogenous IgM^b (Table 1). Thus, Tg-expressing B cells can differentiate to antibody-secreting cells. However, the level of circulating anti-Sm and anti-ssDNA as determined by ELISA is indistinguishable from that of non-Tg littermates (Figure 1).

Anti-Sm and Anti-ssDNA B Cells Populate the Spleen

Based on our earlier L chain pairing analysis (Retter et al., 1995), we expected 2-12H Tg mice to generate anti-Sm, anti-ssDNA, anti-Sm/ssDNA, and non-Sm, non–ssDNA binding B cells. The latter, we presume, are non-autoreactive. To determine whether autoreactive cells are made and reach the periphery, three-color immunofluorescence of spleen cells with anti-B220, anti-IgM^a, and either biotinylated Sm or ssDNA was performed. The percentage of IgM^+ cells that stain with Sm or ssDNA ranges from 15%–35% (Figure 2; see also Figure 4). Sm-binding cells stain with variable intensities indicating that they exhibit a range of affinities for Sm, although there appear to be discreet subpopulations, the largest of which stains approximately 20-fold brighter than non-Sm binding, IgM^+ B cells (Figure 2). ssDNA binding appears to be less variable in intensity. Because the H chain is constant, these differences in binding intensity must be due to the use of different L chains. The percentage of cells in non-Tg littermates that stain with either of these reagents is at least 4-fold lower. Analysis of hybridomas from 2-12H Tg mice is underway to determine the percentage of B cells that bind Sm and ssDNA, but preliminary data indicate that fewer than 10% of anti-Sm B cells bind ssDNA. Thus, it appears that about half or more of the splenic B cells in 2-12H

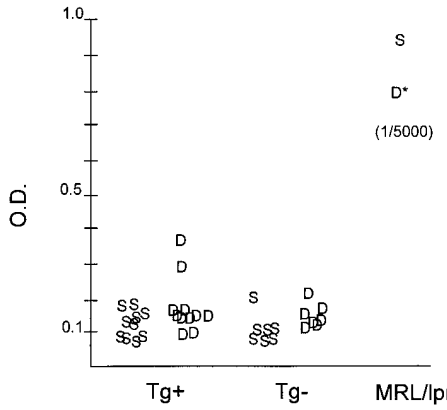

Figure 1. Tg Mice Do Not Have Elevated Levels of Anti-Sm or Anti-ssDNA Antibodies

Sera from Tg and non-Tg littermates were tested by ELISA for anti-Sm (S) and anti-ssDNA (D) antibodies as described in Experimental Procedures. MRL/lpr serum was used as a positive control. Sera from Tg and non-Tg littermates were diluted 1:500, whereas the MRL/lpr control sera was diluted 1:5000 for the anti-ssDNA ELISA and 1:500 for the anti-Sm ELISA. Limit of detection was 0.75 μg/ml IgM for anti-Sm and anti-ssDNA.

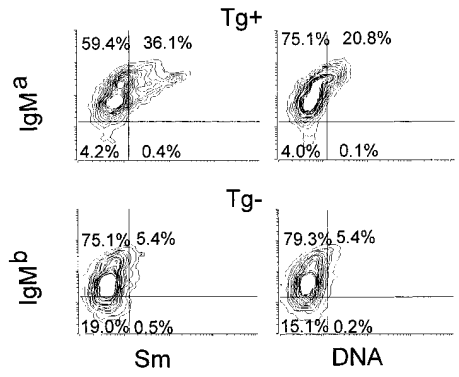

Figure 2. 2-12H Tg Mice Have Large Numbers of Splenic Anti-Sm and Anti-ssDNA B Cells

Spleen cells were prepared from Tg (top) and non-Tg (bottom) mice and costained with IgM^a-PE or IgM^b-PE, respectively, anti-B220-CyChrome and either biotinylated Sm (left) or DNA (right) followed by streptavidin-FITC. Histograms are gated on $B220^+$ cells. Percentages are of $B220^+$ cells. Data shown are representative of at least 10 mice analyzed.

Table 2. Analysis of Hybridomas Generated from 2-12H Tg Mice

Fusion	Fusion Partner	Screened	Anti-Sm	Anti-ssDNA	Non-Sm, Non-ssDNA
1	Ag8.653	51	1 (2%)	0	50 (98%)
2	Ag8.653	90	5 (5.5%)	1 (1.1%)	84 (93%)
3	NSO/1-*bcl-2*	672	168 (25%)	235 (35%)	269 (40%)
4	NSO/1-*bcl-2*	982	359 (36%)	377 (38%)	246 (25%)

Tg mice are autoreactive. The Sm and ssDNA-binding receptors cocap with IgM (data not shown), demonstrating that binding of Sm and DNA is mediated by IgM rather than by a non-Ig molecule, as has been described for DNA (Bennett et al., 1987; Loke et al., 1989; Yakubov et al., 1989; Hefeneider et al., 1992a, 1992b; 1993; Krieg et al., 1995).

Figure 2 also shows that the surface IgM (sIgM) level on autoreactive B cells is approximately 10-fold higher than that on non-Sm, non–ssDNA staining B cells. The sIgM on non-Sm, non–ssDNA binding B cells is not different from that on non-Tg B cells (data not shown). This is in contrast to the receptor down-regulation characteristic of tolerant B cells in hen egg lysozyme (HEL) Tg mice (Goodnow, et al., 1989) but similar to the IgM^{hi} phenotype of anti-ssDNA B cells of 3H9 Tg mice described by Erikson et al. (1991). To exclude the possibility that IgM^{lo} B cells may bind Sm or ssDNA in these mice yet escape detection because the level of surface Ig is too low for detectable staining, we generated a large number of hybridomas from Tg spleen cells and determined the frequency of anti-Sm, anti-ssDNA, and non-Sm, non–ssDNA binding hybridomas (Table 2). Only two of the four fusions performed generated appreciable numbers of anti-Sm and anti-ssDNA hybridomas. Among the hybridomas of these two, 25%–36% secrete anti-Sm antibodies, 36%–38% secrete anti-ssDNA antibodies, and 25%–40% secrete antibodies that bind neither autoantigen (Table 2). The lack of reactivity to Sm or ssDNA by some hybridomas of the latter group was verified by subcloning and assaying at high IgM concentrations (800 μg/ml). These frequencies of anti-Sm and anti-ssDNA are very similar to those determined by flow cytometry (Figure 2), verifying that a large fraction of the B cells in these mice do not bind either autoantigen. Thus, it is unlikely that a significant number of anti-Sm or anti-ssDNA B cells are not detected by flow cytometry. Both the total number of hybridomas and the number of autoreactive hybridomas are significantly elevated using a fusion partner that expresses a transfected *bcl-2* gene (NSO*bcl-2*) relative to a standard fusion partner (Table 2). Although we are not certain why the fusion efficiency is increased, it is possible that an intrinsic property of the anti-Sm and anti-ssDNA B cells permits fusion and survival with a *bcl-2*-transfected fusion partner more so than with the *bcl-2*$^{-}$ fusion partner. The improved fusion efficiency with NSO*bcl-2* may be attributable to tolerance induction (see below) as previously suggested by Ray and Diamond (1994).

As a second confirmation of the frequencies of anti-Sm, anti-ssDNA, and non-Sm, non–DNA binding B cells in the 2-12H Tg repertoire, we examined the splenic B cells of 24-day-old mice. At this age, nearly all splenic B cells are immature B cells, as indicated by the HSA^{hi}, IgM^{hi} phenotype (Allman et al., 1992, 1993) (Figures 3A and 3B), thereby eliminating the level of surface IgM as a variable. We observe that the frequencies of anti-Sm and anti-ssDNA B cells are 35% and 18%, respectively, comparable to those of adult splenic B cells (Figure 3B). Thus, one-half to two-thirds of the B cells in 2-12H Tg mice are autoreactive, and one-third to one-half do not bind Sm or DNA and are presumably nonautoreactive.

The Majority of Autoreactive B Cells Are Immature

To identify the differentiative stage of anti-Sm and anti-ssDNA B cells in adult mice, we examined these cells for heat-stable antigen (HSA or CD24) and CD23 expression, two markers that discriminate between immature and mature follicular B cells. HSA is expressed at high levels by immature B cells but at low levels by mature B cells (Allman et al., 1992, 1993), whereas CD23 is expressed only by mature B cells (Carsetti et al., 1995). 2-12H Tg mice have slightly elevated numbers of HSA^{hi}, immature B cells relative to non-Tg littermates (Figure 4A). Three-color immunofluorescence indicates that the majority of autoreactive B220^{+} B cells in these Tg mice are HSA^{hi} and CD23^{-} (Figures 4B and 4C), consistent with an immature B cell. Essentially all of the HSA^{hi} B cells bind either Sm or ssDNA. About one fourth of the anti-Sm and anti-ssDNA B cells are HSA^{lo} and CD23^{-}, suggesting that some autoreactive B cells have a mature B cell phenotype, although the majority of HSA^{lo} B cells appear to be nonautoreactive. All anti-Sm and anti-ssDNA B cells are CD43^{-} (Figure 4), ruling out assignment to the B-1 cell subset. Anti-Sm and anti-ssDNA B cells are also CD22^{+}, Pgp-1^{+}, Fas$^{lo/-}$, and CD19^{+} (data not shown).

The high IgM and HSA levels of anti-Sm and anti-ssDNA B cells are also characteristic of marginal zone B cells. Marginal zone B cells are mature, noncycling, noncirculating, and long-lived B cells that appear to be involved prominently in T-independent type 2 responses (Snapper et al., 1993). They may also be antigen-selected (Chen et al., 1997). Expression of CD21 is commonly used to distinguish immature and marginal zone B cells (Cyster and Goodnow, 1995); however, Sm and ssDNA interfere with the binding of anti-CD21. Therefore, we could not directly determine CD21 expression levels by autoreactive B cells. However, the percentage of CD21^{hi}, B220^{+} marginal zone B cells is roughly 11% in Tg and 9% in non-Tg mice (data not shown). Therefore, the size of the marginal zone B cell population in these mice is unaltered by Tg expression and is insufficient to account for the autoreactive B cell populations in these mice.

Immature follicular B cells differ significantly in half-life from both mature follicular and marginal zone B cells. Immature B cells have a half-life of less than a week,

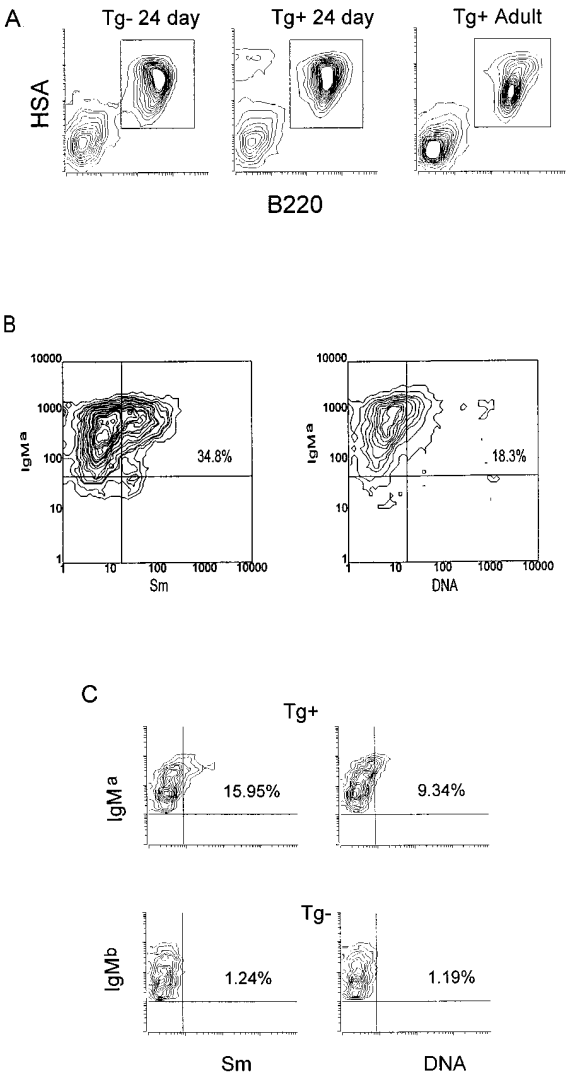

Figure 3. Anti-Sm and Anti-ssDNA B Cells from 24-Day-Old Mice

(A) Two-color immunofluorescence was performed using spleen cells from non-Tg (left), 24-day-old Tg (middle), and adult Tg (right) mice. Staining was done with B220-CyChrome and HSA-PE. Data shown are representative of approximately 16 mice analyzed.

(B) Spleen cells from 24-day-old Tg mice were stained with IgM^a, B220 and either Sm or ssDNA. Histograms are gated on $B220^+$ cells and percentage of autoantigen-binding B cells is given. Data are representative of 16 mice analyzed.

(C) Anti-Sm and anti-ssDNA B cells are present in the bone marrow of 2-12H Tg mice. Gates were set on $B220^+$, IgM^+ populations and analyzed for Sm and ssDNA binding. Data shown are representative of three experiments using three mice each.

whereas the latter B cells have long half-lives of 4 or more weeks (Förster and Rajewsky, 1990; Allman et al., 1993, Chen et al., 1997). To determine whether the half-lives of the autoreactive B cells in 2-12H Tg mice are characteristic of immature or mature B cells, we followed the incorporation of BrdU by HSA^{hi} and HSA^{lo} B cells. Analysis of BrdU incorporation by HSA^{hi} B cells shows that Tg and non-Tg HSA^{hi} B cells incorporate BrdU at equivalent rates, indicating that these B cells are replenished at the same rates (Figure 4C). The half-life, the time at which 50% of the cells are labeled, for HSA^{hi} B cells of both Tg and non-Tg mice is approximately 1 week. Because essentially all of the HSA^{hi} B cells are autoreactive (Figure 4B), these data indicate that the majority of autoreactive B cells have the short half-life of an immature B cell, not mature follicular or marginal zone B cells. Thus, we conclude that anti-Sm and anti-ssDNA B cells of 2-12H Tg mice are immature follicular B cells. Tg HSA^{lo} B cells appear to have a slightly lower half-life compared to non-Tg mice. The significance of this is unknown, but one possible explanation is that the autoreactive HSA^{lo} B cells have shorter half-lives than nonautoreactive HSA^{lo} B cells.

Assignment to the immature subset of B cells predicts that these cells will constitute a high percentage of B cells in the bone marrow. As shown in Figure 3C, approximately 16% of IgM^+ cells in adult bone marrow bind Sm, and approximately 9% bind ssDNA. Although this is a somewhat smaller proportion than observed in the spleen, these data indicate that a large percentage of newly generated B cells bind one or the other autoantigen, thereby excluding accumulation or clonal expansion of these cells as the explanation for the high proportion of autoreactive B cells in the spleen, as reported for marginal zone B cells and B-1 cells (Arnold et al., 1994; Chen et al., 1997).

The anti-Sm and anti-ssDNA B cells do not migrate out of the spleen in significant numbers. As shown in Table 3, lymph nodes of Tg and non-Tg mice are not appreciably different in the percentage of anti-ssDNA B cells, and Tg mice have only a slightly higher frequency of anti-Sm B cells than non-Tg mice. No differences between Tg and non-Tg mice are apparent among peritoneal cells.

Anti-Sm Secretion In Vitro and In Vivo

To assess whether anti-Sm and anti-ssDNA B cells can be activated to secrete anti-Sm and anti-ssDNA antibodies, we LPS-stimulated splenocytes for 3 days and measured the Sm and ssDNA-binding activity of the secreted antibody. LPS stimulation resulted in secretion of anti-Sm antibodies from Tg but not non-Tg mice (Figure 5). Anti-ssDNA antibodies were low even in Tg cell cultures, suggesting they are less responsive to LPS than anti-Sm B cells.

To test the responsiveness of the anti-Sm B cells in vivo, Tg mice were immunized once with murine snRNPs, and anti-Sm reactivity was assayed 10 days later. Figure 6 and Table 4 show that anti-Sm levels are significantly elevated above the preimmune anti-Sm titers. Adjuvant alone did not increase the level (Table 4), and there was no increase in anti-ssDNA levels in these mice (Figure 6, Table 4), arguing that the response is Sm-specific and driven by the snRNP immunogen rather than the result of polyclonal activation.

Discussion

We have demonstrated that 2-12H Tg mice have large numbers of splenic anti-Sm and anti-ssDNA B cells, each accounting for 15%–35% of the repertoire. The majority of these cells are immature follicular B cells

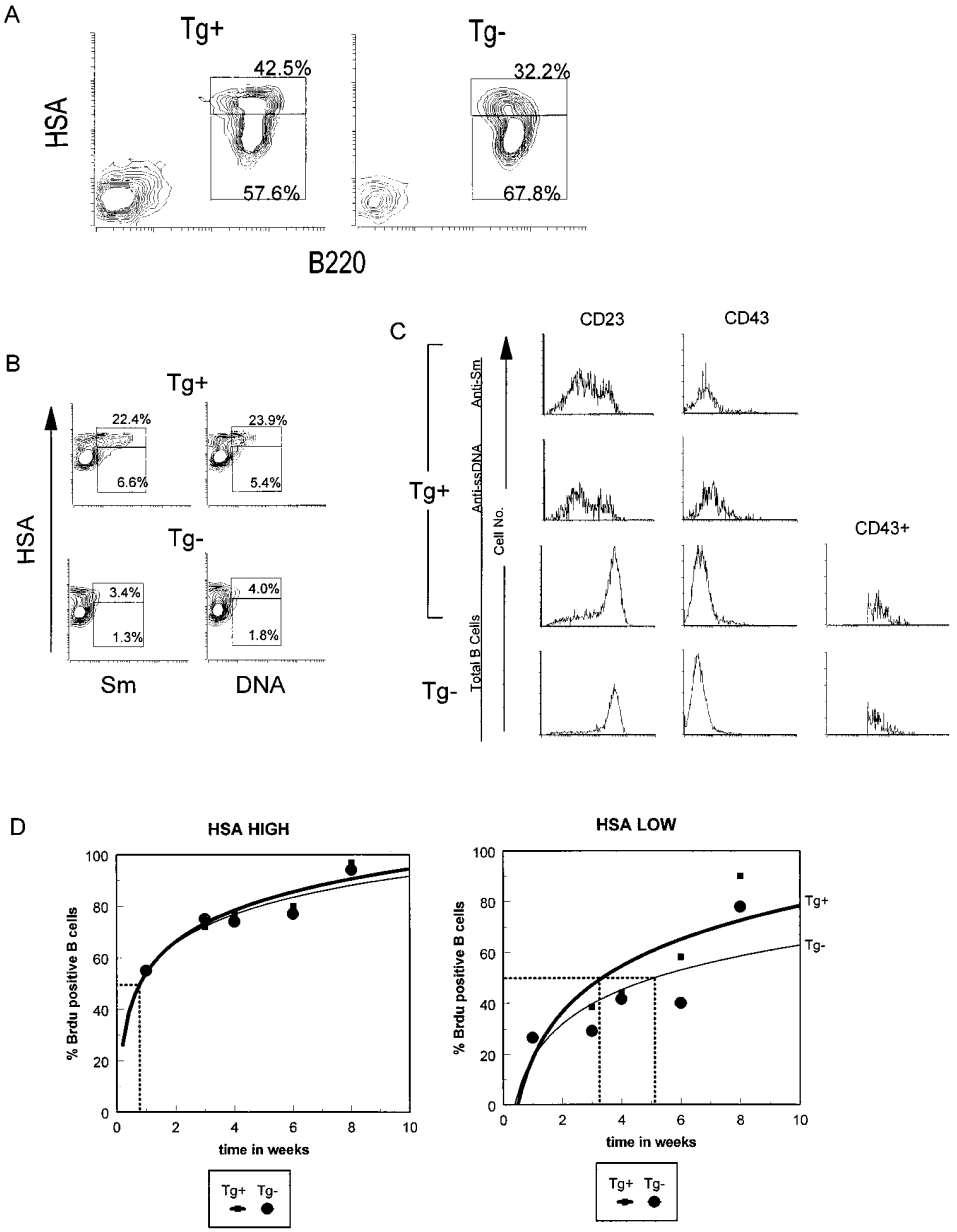

Figure 4. Anti-Sm and Anti-ssDNA B Cells in the Spleens of Adult 2-12H Tg Mice Have an Immature Phenotype

(A) The proportion of B cells that are HSA^{hi} is slightly higher in Tg mice than in non-Tg littermates. Immunofluorescence was performed on spleen cells from Tg and non-Tg littermate mice using B220-CyChrome and HSA-PE. Percentages of B cells that are either HSA^{hi} or HSA^{lo} are given. Data shown are representative of approximately 14 mice analyzed.

(B) Anti-Sm and anti-ssDNA B cells from Tg (top) and non-Tg (bottom) mice were analyzed by three-color immunofluorescence as described in Figure 2. Histograms are gated on B220^{+} cells.

(C) Phenotypic analysis of splenocytes gated on B220^{+} and either Sm^{+}, ssDNA^{+}, or all B cells. Tg splenocytes are presented in the top three rows and non-Tg splenocytes in the bottom row. Immunofluorescence data are representative of three to five experiments using four 3-month-old mice each. Staining was done as described in Figure 2.

(D) BrdU incorporation into HSA^{hi} (left) and HSA^{lo} (right) B cell populations in Tg (thick line) and non-Tg (thin line) mice was performed as described in Experimental Procedures. Dotted lines indicate the time at which 50% of the B cells were labeled. For each time point, two Tg and two non-Tg mice were used; labeling was begun on mice at approximately 2 months of age.

that are short-lived and have an IgM^{hi}, HSA^{hi}, CD43^{-}, and CD23^{-} phenotype, indistinguishable from immature follicular B cells of non-Tg mice. About one fourth of the autoreactive B cells have a mature B cell phenotype and are IgM^{lo}, HSA^{lo}, CD23^{+}, and CD43^{-}. Based on analysis of spleen cells from 24-day-old mice, where all of the cells are essentially newly generated immature B cells, and adult bone marrow, we estimate that a comparable frequency of newly generated B cells are anti-Sm and anti-ssDNA. We presume that this is due to a large number of L chains that can contribute to the binding to one or the other autoantigen when combined to the

Table 3. Percentage of Peripheral Anti-Sm and Anti-ssDNA B Cells among $B220^+$ Cells in Lymph Node and Peritoneum

		Lymph Node		Peritoneum	
	Tg	SM (%)	DNA (%)	SM (%)	DNA (%)
Experiment 1	+	ND	5.5	ND	ND
	+	ND	7.0	ND	12.3
	−	ND	5.2	ND	6.4
Experiment 2	+	4.6	7.2	1.1	1.7
	+	5.2	4.8	1.8	4.0
	−	1.7	5.5	<1	6.1
Experiment 3	+	3.9	2.7	<1	2.2
	+	2.8	4.2	3.4	2.0
	−	1.8	2.4	<1	3.6

ND, not done.

2-12H chain, because we have previously determined that multiple diverse L chains can pair in vitro with the 2-12H chain and bind Sm or ssDNA or both (Retter et al., 1995). Thus, these data argue that the high frequency of these cells in the adult spleen is not due to accumulation or clonal expansion of autoreactive B cells but due to their frequent generation.

Despite the large number of autoreactive B cells, the levels of anti-Sm and anti-ssDNA in circulation are not different from those of non-Tg littermates. Therefore, these cells are not spontaneously activated in normal mice, arguing that they are regulated peripherally. Peripheral regulation of anti-ssDNA B cells has been previously demonstrated (Erikson et al., 1991; Offen et al., 1992; Tsao et al., 1993; Roark et al., 1995), and the presence of anti-Sm B cells in the periphery is predicted from immunization experiments of normal mice with foreign molecular mimics and peptides of Sm (Fatenejad et al., 1993; Mamula et al., 1994; Bockenstedt et al., 1995). The presence of anti-Sm and anti-ssDNA B cells in the periphery is physiologically relevant, because B cells expressing the 2-12 V_H in combination with different V_L are likely to be generated in normal mice; the Tg encodes an unmutated H chain, and the L chains with which it associates should also be unmutated. Thus, the 2-12H Tg mouse is a model of peripheral tolerance to both DNA and protein autoantigens.

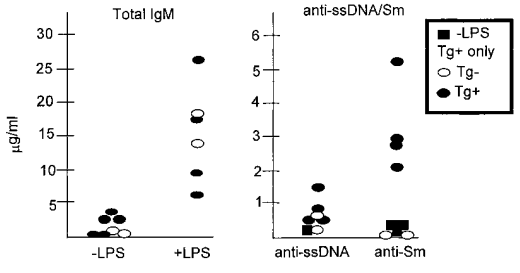

Figure 5. Tg Spleen Cells Can Be Induced In Vitro to Produce Autoantibodies by Activation with LPS

Spleen cells from Tg (filled circles) or non-Tg (open circles) mice were cultured for 72 hr in the presence and absence of 50 μg/ml LPS. Supernatants were harvested and quantitated for IgM (left) and anti-Sm and anti-DNA (right) production using ELISA as described in Experimental Procedures. Anti-Sm and anti-ssDNA reactivity was undetectable in cultures from non-Tg mice in the absence of LPS. Data are presented in μg/ml.

This analysis does not address whether some anti-Sm and anti-ssDNA B cells expressing the 2-12H transgene are deleted in the bone marrow. In this model, the affinity of antibody for Sm or ssDNA is dependent on the associated L chain. Cells that produce an L chain that confers a high affinity for either autoantigen may be centrally deleted upon expression of the IgM receptor, as is the case for high affinity anti-DNA B cells (Chen et al., 1994, 1995).

How anti-Sm and anti-ssDNA B cells are regulated is uncertain. One possibility is that these cells are simply not activated in nonautoimmune mice due to a low affinity for autoantigen, to insufficient levels of endogenous antigen, or to a lack of appropriate T cell help. However, our data suggest a more active process of B cell regulation. We propose that anti-Sm and anti-ssDNA B cells are eliminated from the periphery during the transition to a mature B cell. This possibility is based on the finding that the frequency of autoreactive B cells in the mature B cell subset is less than that in the immature B cell subset (Figure 7). Essentially, the entire immature repertoire in adult 2-12H Tg spleens is autoreactive; over 40%

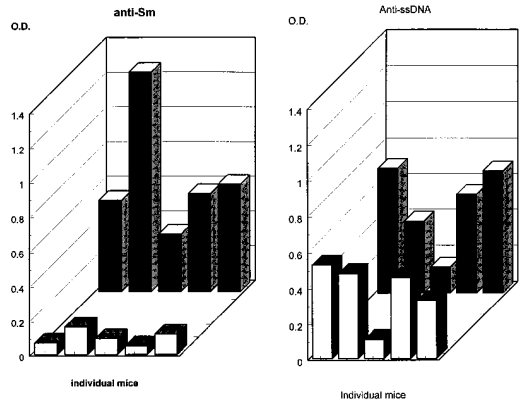

Figure 6. Immunization of Tg Mice with snRNPs Induces Anti-Sm Antibody Production

Tg mice were immunized intraperitoneally with snRNPs in CFA. After 10 days serum was drawn, and anti-Sm (A) and anti-ssDNA (B) reactivity was measured. Data is presented for individual mice before (white bars) and after (black bars) immunization. In the absence of snRNPs, no serum anti-Sm is detected, as with unimmunized mice.

Table 4. Anti-Sm Levels in snRNP Immunized 2-12H Tg Mice

Sera	(μg/ml) Level
Preimmune	ND
CFA	ND
snRNP + CFA	5.15 ± 2.15

Data from 5 mice.
ND, not detectable. The limit of detection is 0.75 μg/ml.

of splenic B cells are immature as defined by the HSA^{hi} phenotype, and the percentage of HSA^{hi}, anti-Sm and anti-ssDNA B cells is also over 40% (Figure 4). Using flow cytometry, only about one fourth of the mature B cell subset is autoreactive (Figure 4); the remainder are non-Sm, non–ssDNA binding B cells. Although some autoreactive B cells may not be detected by flow cytometry because of their low surface IgM level, the number of undetected autoreactive B cells is likely to be low based on the hybridoma analysis (Table 2) and on analysis of B cells from 24-day-old spleens (Figure 3). Thus, the immature and mature B cell repertoires are not equivalent. Deletion of autoreactive B cells from the mature repertoire could account for the lower half-life of HSA^{lo} B cells in 2-12H Tg mice (Figure 4). Which cells become mature is likely to be dependent on affinity for autoantigen, with the highest affinity being excluded from the mature B cell repertoire. We are currently testing this prediction.

A loss of anti-Sm and anti-ssDNA B cells during the transition to the mature B cell subset would be consistent with findings that immature cells are sensitive to deletion by soluble antigen (Stashenko and Klinman, 1980; Klinman et al., 1981; Nossal, 1983; Riley and Klinman, 1986; Carsetti et al. 1995). The phenotype of the deletion-sensitive immature B cell has been further explored by Carsetti et al. (1995). They define a transitional B cell in the bone marrow that is intermediate in development to immature and mature B cells. Transitional cells are sensitive to deletion by surface IgM cross-linking,

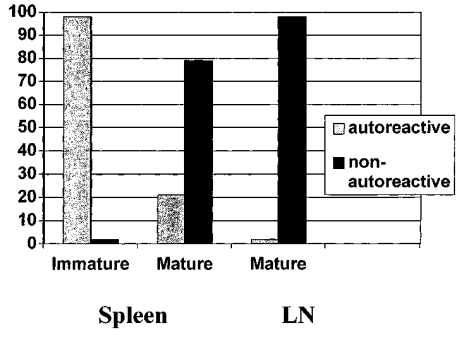

Figure 7. The Immature and Mature B Cell Repertoire of 2-12H Tg Mice Differ in the Proportion of Autoreactive B Cells

In 2-12H Tg mice, the peripheral repertoire of mature B cells differs from immature in that the most of the autoreactive B cell populations are present in lymphoid organs harboring immature B cells. Shown above is the distribution of immature, mature, autoreactive, and nonautoreactive B cells in lymph node (LN) and spleen. Percentage of B cells in each population is represented along the Y axis. Graph represents data compiled from Figure 4B.

whereas immature and mature B cells are not, suggesting that these cells are normally targeted for negative selection in vivo. Bone marrow transitional cells have an approximately 10-fold higher level of surface IgM than immature and mature cells, and they appear to be equivalent to the HSA^{hi} B cells of the spleen (Allman et al., 1993; Carsetti et al., 1995). Thus, anti-Sm and anti-ssDNA B cells in 2-12H Tg mice appear to reach the deletion-sensitive transitional stage of development. High sIgM levels are also seen in 3H9 H chain Tg and 3H9/Vκ8 double Tg mice (Erikson et al., 1991). These mice express an H chain that confers specificity for DNA, and like 2-12H Tg anti-ssDNA B cells, 3H9 Tg anti-ssDNA B cells do not spontaneously secrete antibody. Recent analysis of these mice indicates that the anti-ssDNA B cells are also immature (Mandik-Nayak et al., 1997), suggesting that developmental arrest occurs at the transition from immature to mature B cells as in the 2-12H Tg.

At least some anti-Sm B cells appear to be functional in these mice, because immunization with murine snRNPs induces an anti-Sm response. This is consistent with the lack of sIgM down-regulation among immature anti-Sm and anti-ssDNA B cells. This is the first demonstration of anti-Sm B cell activation in vivo by murine snRNPs, possibly because 2-12H Tg mice have a higher frequency of these cells relative to non-Tg mice (Fatenejad et al., 1993; Mamula et al., 1994; Bockenstedt et al., 1995). This is not a nonspecific activation because anti-ssDNA antibody levels are not altered by this immunization. Whether all anti-Sm B cells are functional is unknown. Possibly, only the mature HSA^{lo} B cells that are Sm^{dull} (Figure 5) are responsible for the observed response, although immature B cells can respond to antigen if provided T cell help (Chang et al., 1991; Allman et al., 1992, 1993). T cells in these mice proliferate in response to peptides of the D protein component of Sm in vitro (M. J. M., unpublished data), suggesting that functional anti-Sm T cells are available for B cell activation. The reason that unimmunized 2-12H Tg mice do not undergo a spontaneous anti-Sm response despite the presence of functional Sm-specific B and T cells may be due to the concentration, form, or accessibility of antigen. Regardless, the anti-Sm B and T cell data together suggest the intriguing possibility that tolerance to Sm is achieved through regulation of the B cell, not the T cell.

We cannot completely exclude the possibility that anti-Sm B cells are anergic, despite the fact that many are IgM^{hi} and that these mice are responsive to snRNP immunization. The immunization with snRNPs may have provided a form of SmD that is able to overcome anergy, similar to the way in which membrane-bound HEL can overcome anergy of anti-HEL B cells (Cooke et al., 1994). Alternatively, only some anti-Sm B cells may be anergic (e.g., those that show a mature phenotype). These may preferentially be those that have a high affinity. Thus, anergy may still play a role in the regulation of anti-Sm B cells. Anergy has been demonstrated to play a role in anti-DNA B cell regulation in 3H9 Tg mice (Erikson et al., 1991; Mandik-Nayak et al. 1997). Whether the anti-ssDNA B cells of 2-12H Tg mice are functional or anergic has not yet been determined. However, anti-ssDNA B

cells secrete little antibody relative to anti-Sm B cells after LPS stimulation in vitro, suggesting that the former are anergic like their counterparts in 3H9 Tg mice (Erikson et al., 1991; Mandik-Nayak et al. 1997).

It is interesting that, although both anti-Sm and anergic anti-HEL B cells enter the periphery, they differ in the expression level of sIgM and responsiveness to antigen (Goodnow et al., 1989, 1991; Adams et al., 1990; Cooke et al., 1994). Unlike anti-Sm B cells, anergic anti-HEL B cells have a 20-fold lower level of sIgM than nonautoreactive cells and are unresponsive to antigen due to an uncoupling of the IgM receptor complex from the normal signal transduction pathway (Bell and Goodnow, 1994; Cooke et al., 1994; Eris et al., 1994). These differences are probably related to the strength of the tolerogenic signal received by the B cell. Tg anti-HEL B cells have an extraordinary affinity for HEL, and in Tg mice also producing soluble HEL, these cells are exposed to high levels of tolerogen, a combination that probably results in a decrease in sIgM levels and loss of signaling function. This is supported by the observation that anti-HEL B cells exposed to a lower level of soluble HEL exhibit a smaller modulation of sIgM (Goodnow et al., 1989; Fulcher et al., 1996). Anti-Sm and anti-ssDNA B cells of 2-12H Tg mice are unmutated and may be of low affinity (Retter et al., 1996), and the level of available Sm and ssDNA may be low. Thus, the degree of anti-Sm receptor engagement in 2-12H Tg mice may be below that necessary to induce receptor down-regulation on newly generated immature B cells.

An interesting parallel can be drawn between the 2-12H chain Tg mice and anti-HEL H chain-only Tg mice in which multiple L chains are available for pairing with transgene-encoded H chains (Cyster et al., 1994; Hartley and Goodnow, 1994). Anti-HEL H chain–only Tg mice have few splenic anti-HEL B cells, and those generated appear to be of low affinity. The anti-HEL B cells fall into discreet subsets depending on the affinity of their sIgM and HEL staining intensity. In the presence of low levels of soluble HEL, B cells of the higher affinity subsets are deleted, while lower affinity subsets remain. With increasing concentrations of HEL, these lower affinity sets of B cells are also lost. Some of the remaining anti-HEL B cells are IgM^{hi}, raising the possibility that they are immature. Whether these cells are functional is not known. These data support the idea that the anti-Sm and anti-ssDNA B cells in 2-12H Tg mice have not received a signal of sufficient strength to induce down-regulation of sIgM, a characteristic of tolerant high-affinity anti-HEL B cells.

We propose, therefore, that contact with Sm, if it occurs in the bone marrow, is not sufficiently strong to alter normal development through the transitional cell stage, and that up until this stage these cells are functional and responsive to antigen and T cell help. However, as transitional cells in the spleen, they are subject to deletion or anergy because the high sIgM level mediates a stronger negative signal, and they are more sensitive to IgM cross-linking-induced cell death than immature B cells (Carsetti et al., 1993, 1995; Norvell et al., 1995; Monroe, 1996; Norvell and Monroe, 1996). Thus, the combination of low-affinity and low-antigen concentration may not permit tolerance induction until a stage late in differentiation at the transition to a mature B cell.

These results demonstrate a peripheral tolerance mechanism acting on anti-Sm and anti-ssDNA B cells. Anti-Sm B cells of three clones using this H chain in association with different L chains were activated in separate MRL/lpr mice undergoing an anti-Sm response (Bloom et al., 1993b). Therefore, the defect in the regulation of anti-Sm and anti-ssDNA B cells in MRL/lpr mice likely involves overcoming peripheral tolerance. Because at least anti-Sm B cells are not anergic, they may be especially susceptible to inappropriate activation. Indeed, 2-12H Tg mice on an MRL/lpr background spontaneously produce high titers of anti-Sm and anti-ssDNA beginning before 1 month of age (S. Ferguson and S. H. C., unpublished data), supporting the conclusion that MRL/lpr mice are defective in this mechanism of peripheral regulation (Rathmell and Goodnow, 1994; Rubio et al., 1996). This is consistent with the observations of others that central tolerance is intact in MRL/lpr mice (Rathmell and Goodnow, 1994; Rubio et al., 1996).

Experimental Procedures

Mice

Tg mice were generated using the rearranged V(D)J segment of hybridoma 2-12H (Bloom et al., 1993b) cloned upstream of the Igh^a $C\mu$ gene (Retter et al., 1995). Purified insert was injected into $(C57BL/6 \times SJL)F_1$ blastocysts and then implanted into pseudopregnant mice. Two Tg founders were obtained and further backcrossed to C57BL/6 or C.B17 mice for eight or more generations. Transmission of the transgene was verified by polymerase chain reaction analysis of tail DNA using an oligonucleotide identical to a sequence 470 bp 5′ of the 2-12 V_H exon (5′-CAGACTAAGGCCAAATATCAACT GAGAGA-3′) and an oligonucleotide complementary to a sequence 3′ of J_H4 (5′-CAGGCTCCACCAGACCTCTCTAGA-3′) (Retter et al., 1996). DNA was prepared by incubating tail snips in 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% SDS (modified STE) buffer (Drews et al., 1994) with 1 μg/μl proteinase K at 55°C overnight. For amplification 2 μl of tail digest was added to 50 μl of polymerase chain reaction mix consisting of 1.25 mM dNTP's, 1.5 mM $MgCl_2$, each primer at 2 μM, 5 U Taq polymerase (Promega, Madison, WI), and 5 μl of 10× polymerase buffer (Promega). Reactions are run for 30 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, followed by 10 min at 72°C for 1 cycle in a Perkin-Elmer/Cetus thermocycler. Several founder lines $(C57BL/6 \times CB.17)$ F_2 were generated, and one is presented here in detail. Animals are age- and sex-matched in all experiments and are housed in a conventional facility at UNC.

Serum ELISA

Detection of anti-DNA and anti-Sm by ELISA was done as previously described (Eisenberg et al., 1982; Bloom et al., 1993b). Briefly, 96-well PVC plates were coated with antigen in borate-buffered saline (BBS) (Mishell and Shiigi, 1980), washed, and blocked with BBS/BSA/Tween(BBT). All washes were done with BBS. Mouse sera were serially diluted in BBT then added to antigen coated plates in duplicate. IgM was quantitated by adding sera to plates coated with polyclonal goat anti-mouse IgM (Southern Biotechnology, Birmingham, AL) and detected with either biotinylated anti-IgM^a (HB100) $F(ab)_2$ fragments (a kind gift from P. Cohen) or intact anti-IgM^a or anti-IgM^b (Pharmingen, San Diego, CA), followed by streptavidin-alkaline phosphatase (Southern Biotechnology). Assays were developed with 1 mg/ml *p*-nitrophenyl phosphate (Sigma, St. Louis, MI) in 0.01 M diethanolamine and the OD_{405} determined. Serum titers of IgM were determined by generating a standard curve from serial dilutions of TEPC 183 (IgM^a) or CBPC17 (IgM^b) in each assay.

LPS Stimulation

Spleen cells were harvested aseptically, washed, and resuspended at 1×10^6 cells/ml in complete DMEM-H and incubated for 72 hr in

10% CO_2 in the presence or absence of 50 μg/ml LPS. Supernatants from triplicate cultures were tested by ELISA for anti-Sm, anti-ssDNA, and IgM production.

Immunofluorescence

Bone marrow was extracted from femurs of the two hind legs. Spleens and lymph nodes were collected and made into single-cell suspensions by crushing between the frosted ends of glass slides. Cells from the peritoneum were collected by peritoneal lavage. All cells were prepared and washed in complete Hanks' balanced salt solution (HBSS). Following lysis of red blood cells using 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2–7.4), cells were washed in incomplete HBSS, and staining was carried out at 4°C. Cells were incubated with anti-F_CR antibody (2.4G2) (a gift of G. Haughton) for 10 min at 4°C prior to addition of labeled antibodies. Cells were stained in complete HBSS (incomplete HBSS with 3% fetal calf serum) with anti-IgM-phycoerythrin (PE), anti-IgM^a-PE, anti-IgM^b-PE, anti-IgM^a-fluorescein isothiocyanate (FITC), anti-IgM^b-FITC, anti-CD24-PE (HSA), anti-B220-CyChrome (CD45), anti-CD95-PE (Fas), anti-$CD23^-$PE, and anti-CD44-PE (Pgp-1) purchased from Pharmingen.

To identify autoreactive B cells, spleen cells were incubated with either a 40-base oligonucleotide degenerate at every position and biotinylated on the 3′ terminal nucleotide during synthesis (GIBCO BRL, Gaithersburg, MD) or with calf thymus/spleen Sm (Immunovision, Springdale, AR) that was biotinylated. Both ssDNA and Sm-binding B cells were detected by FITC-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) and flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA). Biotinylation of Sm was carried out in 0.1 M $NaHCO_3$ using biotin-X-NHS (Calbiochem, San Diego, CA) at 4°C. Excess biotin was removed by dialysis against phosphate-buffered saline. Immunoglobulin-specific binding of each of these reagents was verified by colocalization experiments. Briefly, surface Ig was costained with Sm or DNA and anti-IgM-PE. Capping was done at 37°C, and samples were taken at 5 min intervals, fixed in 1% paraformaldehyde, then washed and incubated with streptavidin-FITC. Samples were visualized and photographed using a fluorescent microscope. Approximately 1×10^6 cells were used per experiment.

Hybridomas

Mouse spleen cells were aseptically harvested, and a single-cell suspension was prepared. Cells were cultured for 16 hr in 25 μg/ml LPS as described above. Fusion partners P3X63-Ag8.653 and NSO*bcl-2* (Ray and Diamond, 1994) were selected in complete DMEM-H supplemented with 8-azaguanine. Just prior to fusion, all cells were washed in incomplete DMEM-H and then resuspended in 50% PEG 1500 (Boehringer Mannheim Biochemicals, Germany) and incubated at 37°C. Following fusion, cells were resuspended in HAT medium supplemented with 10% origin hybridoma cloning factor (Igen, Rockville, MD) and cultured in 96-well plates. Supernatants were tested for anti-Sm, anti-ssDNA, and IgM^a by ELISA as described above.

BrdU Labeling

Adult mice were BrdU-labeled in vivo using the method of Allman et al. (1993). Briefly, 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was administered in drinking water at 0.5 mg/ml with 1 mg/ml dextrose (Mallinckrodt, Paris, KY) continuously for 1–8 weeks. At each time point, mice were sacrificed and spleen cells prepared for staining with HSA-PE and B220-CyChrome as described above. Subsequent permeabilization followed by treatment with DNase (Sigma, St. Louis, MO.) and staining with anti-BrdU-FITC (Becton Dickenson, San Jose, CA) allowed use of FACS analysis to assess the fraction of B cells BrdU-labeled.

Cell Sorting

Spleen cells were stained with anti-HSA-PE and anti-B220-FITC. $B220^+$ HSA^{hi} and $B220^+HSA^{lo}$ cells were sorted using a MoFlo flow cytometer (Fort Collins, CO) according to the level of HSA expression. Cells from each fraction were cultured in complete DMEM-H in 10% CO_2 in triplicate at $.5 \times 10^6$ cells/ml in the presence or absence of 50 μg/ml LPS. Supernatants were collected at 72 hr and quantitated by ELISA for IgM^a production and then tested for anti-ssDNA and anti-Sm reactivity as described above.

Immunizations

Mice were immunized intraperitoneally with 100 μg murine snRNPs in complete Freunds adjuvant (CFA) (Sigma) or CFA alone as described previously (Fatenejad et al., 1993; Mamula et al., 1994; Bockenstedt et al., 1995). Serum was collected before and at 10 days after immunization, at which time IgM^a, anti-Sm, and anti-ssDNA production was quantitated by ELISA as described above.

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